REMARKS

The Specification is amended to have a new title that is descriptive. In addition the Specification is amended to contain sequence identifiers for sequences disclosed in Figures 1b, 6 and 7. A Substitute Sequence Listing and an accompanying Sequence Submission Statement are filed herewith. Claims 9-18 are currently pending in the application. New claims 17 and 18 have written support in the Specification as filed as follows:

Claim	Support
17	Page 6, lines7-12, 15-16 and 20-21
18	Page 6, lines 22-25

No new matter has been introduced herewith. The following addresses the substance of the Office Action.

Rejection under 35 U.S.C. § 101

Claims 9-12, originally written as depending form on Claim 1 were rejected as reading on a product of nature. However, the claimed nucleotide encoding "a secretory or membrane-binding chimeric protein composed of an energy-generating protein and an energy-receiving protein linked one another wherein energy transfer can arise between the energy-generating protein and the energy-receiving protein" can only be made by recombinant DNA technology, as such secretory or membrane-binding chimeric proteins do not exist in nature. Thus, the disclosed chimeric proteins or nucleic acids encoding them do not read on a product of nature. Accordingly, the Applicants respectfully request removal of the rejection.

Rejections under 35 U.S.C. § 112, First Paragraph

Written Description Requirement

Claims 9-12 were rejected under 35 U.S.C. § 112, first paragraph as lacking written description in the Specification. The polynucleotide of Claim 9 was alleged to be devoid of a structure and was said to only be defined by the activity of encoding a protein. Since the claims are directed to a genus of DNA and proteins, the Examiner concluded that the skilled artisan could not envision the detailed chemical structure of the chimeric polypeptides encoded or the encoding nucleic acid absent guidance. The applicants have amended Claim 9 to include the limitation wherein the chimeric protein is composed of *Vargula* luciferase or *Cypridina* luciferase and YFP. By this amendment, the energy-generating protein is restricted to *Vargula* luciferase or *Cypridina* luciferase and an energy-receiving protein is restricted to YFP (yellow

fluorescent protein). Thus, the amended claims disclose the invention with sufficient specificity so that one of skilled in the art would know the detailed chemical structure of the chimeric polypeptides encoded or the encoding nucleic acid without any additional guidance.

Moreover, in the Examples of the specification, it is described how to construct the chimeric protein composed of *Vargula* luciferase and YFP, and it has been confirmed that an energy transfer arises between *Vargula* luciferase and YFP. Therefore, the claimed polynucleotides encoding the disclosed chimeric proteins are fully described in the specification.

Additionally, as for the chimeric protein composed of *Cypridina* luciferase and YFP, *Cypridina* luciferase is exemplified as an energy-generating protein in the specification on page 10, lines 4-14. As is known in the art, *Cypridina noctiluca* is a closely-related species of *Vargula hilgendorfii* (see page 2, line 27 of the specification), and luciferases derived from these two marine ostracods have many properties in common. Referring to the attached reference by Nakajima et al. 2004 *Biosci Biotechnol Biochem* **68**:565-570, *Cypridina* luciferase is similar in sequence to *Vargula* luciferase. Namely, the amino acid homology is 83.1%, and important components forming the active structure of the enzyme are conserved in both luciferases (see from page 567, right column to page 568; Fig.2; and Table 1 of Nakajima et al. *supra*). In addition, the Applicant submits herewith a declaration of an additional experiment in order to show that *Cypridina* luciferase can also be used as an energy-generating protein of the present invention.

Thus, given the amendments to the claims and the known structure-function information relating to *Vargula* luciferase or *Cypridina* luciferase and YFP, the claims are in compliance with the written description requirement of 35 U.S.C. § 112, first paragraph and removal of the rejection is respectfully requested.

Enablement Requirement

Claims 9-12 were rejected as allegedly not being enabled, because the Specification, while being enabling for a specific DNA encoding a specific protein, does not reasonably provide enablement for any DNA or protein encoded. In view of the amendments to the claims and the disclosure in the Examples of the Specification for how to construct the chimeric protein composed of *Vargula* luciferase and YFP, the claimed polynucleotides encoding the disclosed chimeric proteins are fully enabled by the Specification so that one of skill in the art would be able to practice the present invention. Accordingly, the subject matter of the amended claims

meets the enablement requirement of 35 U.S.C. § 112, first paragraph and removal of the rejection is respectfully requested.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, the Applicants are not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. The Applicants reserve the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that the Applicants have made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

In view of the Applicants' amendments to the Specification and the Claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 28 December 2007

By: 

Raymond D. Smith
Registration No. 55,634
Agent of Record
Customer No. 20,995
(805) 547-5580

4625341
120607

Biosci. Biotechnol. Biochem., 68 (3), 565–570, 2004

cDNA Cloning and Characterization of a Secreted Luciferase from the Luminous Japanese Ostracod, *Cypridina noctiluca*

Yoshihiro NAKAJIMA,[1] Koji KOBAYASHI,[1] Kazutoshi YAMAGISHI,[1] Toshiteru ENOMOTO,[2] and Yoshihiro OHMIYA[1,†]

[1]*Cell Dynamics Research Group, The Special Division for Human Life Technology, National Institute of Advanced Industrial Science and Technology (AIST), 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan*
[2]*ATTO Co., 1-25-23 Hongo, Bunkyo-Ku, Tokyo 113-8425, Japan*

Received September 2, 2003; Accepted December 1, 2003

A secreted luciferase from the marine ostracod, *Vargula hilgendorfii*, is a useful tool for gene expression assays in living mammalian cells. We have cloned the cDNA of a new secreted luciferase from the ostracod *Cypridina noctiluca*, which inhabits the coast of Japan. *C. noctiluca* luciferase consists of 553 amino acid residues with a molecular mass of 61,415 Da, as deduced from the nucleotide sequence. The homologies of nucleotide and amino acid sequences with *V. hilgendorfii* luciferase are 79.2% and 83.1%, respectively. *C. noctiluca* luciferase can expressed in and secreted from cultured mammalian cells. The characteristic properties of expressed *C. noctiluca* luciferase are similar to those of *V. hilgendorfii* luciferase. However, the activity of *C. noctiluca* luciferase in culture medium is much higher than that of *V. hilgendorfii* luciferase, suggesting that *C. noctiluca* luciferase is a highly potent reporter enzyme for real-time and continuous monitoring of gene expression in living cells.

Key words: bioluminescence; luciferase; ostracod; reporter gene; secretion

The bioluminescence mechanism of the luminous ostracods has been studied only for the Japanese ostracod, *Vargula hilgendorfii* (Fig. 1, left). This organism produces a bright blue light by ejecting luciferin (substrate) and luciferase (enzyme) into the seawater from the upper-lip gland.[1] The luminescence is produced by a simply and highly specific enzyme–substrate reaction, which involves the oxidation of luciferin by molecular oxygen, catalyzed by luciferase (Scheme 1).[1–3] The products of the reaction are blue light (hν 465 nm), oxyluciferin, and carbon dioxide.[2] Excited-state oxyluciferin bound to luciferase is the emitter in the reaction.[4] The cDNA encoding

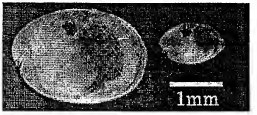
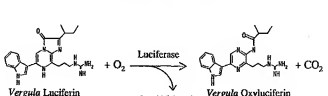


Fig. 1. Luminous Marine Ostracod, *V. hilgendorfii* (left), Commonly Known as "Umi-hotaru" or "Sea-firefly", Is Closely Related to the Species *C. noctiluca* (right).
Scale bar is 1 mm.



Scheme 1.

† To whom correspondence should be addressed. Fax: +81-727-51-9628; E-mail: y-ohmiya@aist.go.jp

Abbreviations: Da, daltons; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction: pfu, plaque-forming units; RLU, relative light units; SDS, sodium dodecyl sulfate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol

V. hilgendorfii lucifcrase (VhL) has been previously cloned, and sequence analysis revealed that this luciferase consists of 555 amino acid residues with a molecular mass of 62,171 Da, as deduced from the nucleotide sequence.[5] VhL contains a secretion signal that results in its secretion from transfected cultured mammalian cells, although the precise cleavage site has not been identified.

Reporter assay systems using luciferases have been widely used to monitor gene expression, because they are highly sensitive and strictly quantitative. In particular, secreted luciferase is used for the real-time and continuous monitoring of gene expression because promoter activity can be measured without destroying the cells.[6] In the previous study, stable transformants of Chinese hamster ovary cells carrying the VhL gene secreted the luciferase, and this secretion from discrete sites on the cell surface could be monitored in real-time using an image-intensifying technique.[7] Recently, the promoter activity of growth hormone in rat pituitary adenoma GH3 cells was monitored continuously for 24 h using VhL in living cells.[8] Thus, VhL can be applied widely in the field of life-science technology. However, a highly active type of the secreted reporter enzyme could be of more benefit to monitor gene expression of a less active promoter, and a slight change of expression level in small number of cells or tissue. We therefore sought a higher active type of secreted luciferase from other ostracods.

Luminous marine ostracods are found in only two genera, *Vargula* and *Cypridina*.[9] The luminous genus *Vargula* is benthonic, and many species inhabit the Gulf of Mexico, the coast of California, and the Caribbean Sea. On the other hand, the luminous genus *Cypridina* is planktonic and a few species are found throughout the world.[9-11] One *Vargula* species (*V. hilgendorfii*) (Fig. 1, left) and two *Cypridina* species (*C. noctiluca*, Fig. 1, right and *C. innermis*) are found in Japan, where they are commonly known as "Umi-hotaru" or "Sea-firefly". Although the biological and ecological traits of luminous ostracods differ, all of them emit a bright blue light when the organisms are exposed to physical or chemical stimulation. In our preliminary work, an extract of *C. noctiluca* cross-reacted with luciferin from *V. hilgendorfii*, suggesting that a similar type of luciferase also exists in *C. noctiluca*.

In this study, we cloned the cDNA of *C. noctiluca* luciferase (CnL) and expressed in cultured mammalian cells. Furthermore, characteristic properties of CnL were compared with those of VhL. Although the almost enzymatic properties of CnL are similar to those of VhL, CnL enzyme shows a higher activity than VhL, allowing more sensitive monitoring of gene expression in a wide variety of mammalian cells.

Materials and Methods

Reagents. The following reagents were obtained from commercial sources: restriction enzymes (Takara, Kyoto, Japan; New England BioLabs, Beverly, MA; Nippongene, Toyama, Japan), sodium dodecyl sulfate (SDS), 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), dithiothreitol (DTT), sodium chloride (Wako Pure Chemicals, Osaka, Japan; Nacalai Tesque, Kyoto, Japan), Isogen (Nippongene, Toyama, Japan), Oligodex-dT30 (Takara).

Construction of cDNA library. C. noctiluca (1.5 g wet weight) was collected at Shimoda (Izu Peninsula, Japan), frozen quickly in liquid nitrogen, and stored at −80°C until use. Total RNA was prepared with Isogen according to the manufacturer's instructions. Poly(A)$^+$ RNA was isolated using Oligotex-dT30 and cDNA synthesized using a Timesaver cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Ligation of cDNA into the λZAPII vector (Stratagene, La Jolla, CA) was performed for 12 h at 4°C, and the products were packaged using the GigapackIII Gold Packaging Kit (Stratagene). The packaged cDNA was transfected into *Escherichia coli* XL1-Blue, yielding 2.3×10^5 plaque-forming units (pfu). The cDNA library was amplified to 2.9×10^8 pfu and screened.

Isolation and sequence determination of cDNA clone. An aliquot of the *C. noctiluca* cDNA library, containing about 8.0×10^4 plaques, was screened using nylon membranes (Amersham Pharmacia Biotech), following the manufacturer's directions. A 1.3-kbp *Xba*I-digested internal fragment of VhL cDNA was labeled with alkaline phosphatase using the Alkphos Direct Labeling Kit (Amersham Pharmacia Biotech) and used as a probe. The membrane was hybridized for 12–14 h at 55°C, then washed twice with the first wash solution (2 M urea, 0.1% [w/v] SDS, 50 mM sodium phosphate, 150 mM NaCl, 1 mM $MgCl_2$) for 10 min at 55°C, and then twice with the second washes solution (50 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$). Positive plaques were detected using an ECL Kit (Amersham Pharmacia Biotech), and rescreened. The insert was excised as a pBluescript phagemid using ExAssist helper phage (Stratagene). The nucleotide sequence of the isolated cDNA was determined using BigDye Terminator and DNA sequencer model 310 (Applied Biosystems). To determine the full-length cDNA sequence of CnL, PCR was done with LA Taq DNA polymerase (Takara), using the *C. noctiluca* cDNA library as template and the specific primer (CL-1R) 5′-TTGAACTTGACGACCAGAGC-3′ and the vector primer (M13 Reverse) 5′-GTAAAACGACGGC-CAGTG-3′, with the thermal profile: 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min 30 s. A 100-fold dilution of the PCR product was used as the template for the second PCR, which was done with the specific primer (CL-2R) 5′-GTAGATGGAAGTG-TTCTGGG-3′ and the vector primer (T3) 5′-AAT-TAACCCTCACTAAAGGG-3′, using the same cycling profile. The single amplified product was cloned into

pCR2.1–TOPO (Invitrogen, Carsbad, CA), and the DNA sequence was determined.

Cell culture. NIH3T3, rat2, A549, COS-7, and HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (ICN Biomedicals Inc., Costa Mesa, CA) at 37°C with 5% CO_2.

Expression of luciferase in mammalian cells. To construct a CnL expression vector for mammalian cells, the full-length cDNA was PCR-amplified from the cDNA library using primers (CL-N) 5′-ATGAAGACC-TTAATTCTTGC-3′ and (CL-C) 5′-CTATTTGCATT-CATCTGGTAC-3′, with the same thermal profile described above. The amplified PCR product was ligated into pCR2.1–TOPO. CnL was excised with *Bam*HI and *Not*I, and ligated into the *Bam*HI/*Not*I site downstream of the cytomegalovirus (CMV) promoter in the mammalian expression vector, pcDNA3 (Invitrogen), to produce pcDNA–CL. To construct a VhL expression vector, the full-length cDNA was also amplified from pSV2-VL[9] by PCR using primers (VL-F-*Not*I) 5′-GCG-GCCGCATGAAGATATAATTCTGTCTG-3′ (start codon was underlined) and (VL-R-*Not*I) 5′-GCGGCC-GCTTATTGACATTCAGGTGGTACT-3′, using the same thermal profile. The amplified product was digested with *Not*I, and ligated into the *Not*I site of pcDNA3, to produce pcDNA-VL. For expression of CnL and VhL in cultured mammalian cells, 5×10^4 cells were plated in each well of a 24-well plate 1 day before transfection. Fifty ng of the vector DNA was transfected into the cells using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. After 3 h of incubation, the transfection medium was replaced with 300 μl of serum-free DMEM and the transfected cells were cultured further for the expression of the luciferase until harvest time.

Measurement of luciferase activity. CnL and VhL activities were measured by mixing 50 μl of the culture medium from luciferase-expressing cells or luciferase-expressing-cell extracts and 50 μl of 280 nM luciferin in 10 mM Tris-HCl (pH 7.4), which was extracted and purified from *V. hilgendorfii*, using an AB-2200 luminometer (ATTO, Tokyo, Japan). The activity was expressed in relative light units (RLU).

K_m and V_{max} values and thermostability of luciferases. To estimate the K_m and V_{max} of CnL and VhL, 50 μl of 5–250 nM luciferin in 10 mM Tris-HCl (pH 7.4) were injected into 50 μl of luciferase-expressing culture medium at 25°C. The values were calculated from Lineweaver–Burk plots of the reciprocal of RLU *versus* substrate concentration. To estimate the thermal stability of CnL and VhL, luciferases-expressing-culture medium were incubated until 60 h at 37°C. The half-life time was estimated by the remaining activities as a thermal stability.

Bioluminescence spectrum measurement. Bioluminescence spectra were recorded using an AB1850 spectrophotometer (ATTO). To assess the bioluminescence spectrum of CnL, 15 μl of the culture medium was mixed with 15 μl of 280 nM luciferin in 10 mM Tris-HCl (pH 7.4). All spectra were corrected for the photosensitivity of the equipment, and normalized.

Sequence analysis. A search of the non-redundant database of the National Center of Biotechnology Information (NCBI) was done using the program Gapped Blast. The molecular mass and isoelectric point (pI) of VhL and CnL were calculated using Genetyx software ver. 6.0.

Continuous measurement of CnL activity. The expression vector pcDNA–CL was transfected into NIH3T3 cells, which were seeded in a 48-well plate. After 2 h of transfection, the medium was replaced with 250 μl of serum-free DMEM (time 0). The culture medium was collected and the cells were disrupted in 250 μl of PBS at 2-h intervals until 10 h and additionally at 12-h interval until 48 h.

Results and Discussion

Habitat of C. noctiluca

C. noctiluca (Fig. 1, right) is a pelagic species found on the Pacific Ocean side of Japan, especially abundant in Hachijo Island or Misaki in the Miura Peninsula of Japan.[1,11] *C. noctiluca* is one-third the size of *V. hilgendorfii* (Fig. 1, left), and usually floats on the surface of the sea at night, whereas *V. hilgendorfii* is benthonic, living in coastal sands. We collected them using a plankton net. In this study, *C. noctiluca* was collected at Shimoda during the period from October 2000 to February 2001.

Nucleotide and amino acid sequences of CnL

C. noctiluca emits a blue light and its extract cross-reacted with anti-VhL antibody (data not shown). To isolate the cDNA encoding CnL, a *C. noctiluca* cDNA library was screened with a VhL cDNA fragment as probe, and six positive clones were isolated. DNA sequence analysis showed that all the positive clones showed high homology with part of the 3′ region of VhL (about 700 bp) containing the poly(A) tail. A clone containing the 5′ region of CnL was isolated by PCR from the cDNA library, and then the full-length sequence of CnL was determined. The complete nucleotide sequence of CnL cDNA contains an open reading frame of 1,659 nucleotides encoding a protein of 553 amino acids (Fig. 2, upper lane). The molecular mass and pI were calculated to be 61,415 Da and 4.59, respectively (Table 1). CnL has a sequence similarity to VhL (Fig. 2, asterisks); the amino acid homology is

```
  1 MKTLILAVALVYCATVHCDD-CPYEPDPPNTVP---TSCKAKEGGCIDSSCGTCTRDILS
  1 MKIIILSVILAYCVTDNCDACPVEAEPPSSTPTVPTSCKAKEGGCLDTRCATCKRDILS
    ** * * * ** * *** ** * **  *  ********** * ** *****

 57 DGLCENKPGKTCCRMCDYVIECRVEAAGWFRTFYGKRFQFPGCTYVLGQGTKGGDWKVS
 61 DGLCENKPGKTCCRMCDYVIECRVEAAGYFRTFYGKRFNFQEPGKYVLARGTKGGDWSVT
    ****************************** ********* ****** ******** *

117 ITLENLDGTKGAVLTKTRLEVAGDIIDIAQATENPITVNGGADPIIANPYTIGEVTIAVV
121 LTMENLDGQKGAVLTKTTLEVAGDVIDITQATADPITVNGGADPVIANPFTIGEVTIAVV
    * ***** ******** ****** *** *** ********* **** **********

177 EMPGFKKVIEFFKLIVIDILGGRSVRIAPDTANKGMISGLCEDLKMMEDTP-DFTSDPEQ
181 EIPGFKKVIEFFKLIVIDILGGRSVRIAPDTANKGLISGLCENLEMN-DADDPTTDADQ
    * ********** ********************************* *** ** * *  *

236 LAIQPKINQEFDGCFLYGNPDDVAYCKGLLEPYKDSCRNPINFYYYTISCAFARCMGGDE
240 LAIQPNINKEFDGCPFYGNPSDIEYCKGLMEPYRAVCRNNINFYYYTLSCAFAYCMGGEE
    ***** ** ***** ***** * * ***** *** *** ******** ***** **** *

296 RASHVLLDYRETCAAPETRGTCVLSGHTFYDTFDKARYQFQGFCKEILMAADCFWNTWDV
300 RAHHVLFDIVETCAAPETRGTCVLSGHTFYDTFDKARYQFQGFCKEILMAADCHWNTWDV
    ** *** ** ********************************** ********** *****

356 KVSHRNVDSYTEVEKVPIRKQSTVVELIVDGKQILVGG-EAVSIPYSSCGKGIYWQDGDI
360 KVSHRDVESYTEVEKVPIRKQSTVVDLIVDGKQVKVGGVD-VSIPYSSSGKGIYWQDGDI
    ***** * ******** ******** ******** ***  ******** *********

415 LTTAILPEALVVKFNFKQLLVVHIRDPFDGKTCGICGNYNQDFSDDSFDAEGACDLTPNP
419 LTTALIPEALVVKFNFKQLLVVHIRDPFDGKTCGICGNYNQDSTDDFFDAEGACDLTPNP
    ******************************************  ** ******* *****

475 PGCTEEEQKPEAERLCNSLFAGQGSDLDQKCNVCAKPDRVECCHYEYCLRGQQGKCDCHAWEF
479 PGCTEEEQRPEAERLCNSLFDSSI--DEKCNVCAKPDRIACCHYEYCLRGQQGKCDCHAWEF
    **************** **          * ***** ***  ********************

535 KKECYIKHGDTLEVPDECK 553  C. noctiluca
537 KKECYIKHGDTLEVPPECQ 555  V. hilgendorfii
    *************** **
```

Fig. 2. Alignment of the Deduced Amino Acid Sequence of CnL (upper, Accession No. AB159608) and VhL (lower, Accession No. M25666). Amino acids have been abbreviated using the standard single-letter code. Identical amino acid residues are indicated by asterisks. Putative *N*-linked glycosylation sites are showed by shadowed boxes. Conserved cysteine residues are indicated by open boxes.

Table 1. Comparison of Characteristic Properties of CnL and VhL

	C. noctiluca luciferase	*V. hilgendorfii* luciferase
Number of amino acid residues	553	555
Molecular mass (Da)	61,465	61,693
pI	4.59	4.51
K_m (μM)	30.0	11.5
V_{max} ($\times 10^5$ RLU/s)	2.20	2.20
Maximum wavelength of emission (nm)	465	465
Half-life time (h) at 37°C	53	50

83.1%. Interestingly, the positions of all 34 cysteine residues (Fig. 2, open boxes) and the locations of the glycosylation sites (Fig. 2, shadowed boxes) are completely conserved in both luciferases. It has been reported that no free sulfhydryl groups were detected in VhL, and the cysteine residues presumably exist as intra-molecular disulfide bridges.[12] Cysteine residues are one of the important components forming the active structure of the enzyme. In fact, both purified VhL and CnL have a tendency to decrease in activity after the addition of DTT (data not shown). Therefore, cysteine residues could play an important role in the formation of the active structure and in the enzymatic activity of these luciferases. CnL contains two *N*-glycosylation sites at the same positions as VhL (Fig. 2). Although the role of *N*-linked oligosaccharide bound to both luciferases has not been clarified, they are thought to play an important role in the stabilization, transport, and secretion of the protein in cells.[13] CnL is the second enzyme to be discovered as the secreted- and oligosaccharide-bound-luciferase. These characteristics of both luciferases may be important in maintaining their activity.

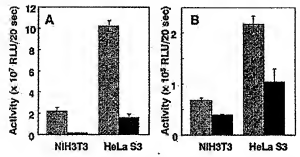


Fig. 3. The Activity of CnL and VhL in the Culture Medium and the Extracts of Cultured Mammalian Cells.
(A) CnL activity in the medium (gray bars) and the cell extracts (black bars) of NIH3T3 and HeLa S3 cells. (B) VhL activity in the medium (gray bars) and the cell extracts (black bars) of NIH3T3 and HeLa S3 cells. Activity was measured after incubations of 25 h. Results are given as means±S.D. ($n = 3$).

Comparisons between properties of two luciferases

To confirm whether CnL is expressed in and secreted from mammalian cells, the expression plasmid pcDNA–CL was transfected into COS-7, NIH3T3, HeLa 3S, A549, and rat2 cells, and luciferase activity was confirmed in the culture medium and in the cells (data not shown). The results indicate that CnL can be expressed in and secreted from cultured mammalian cells. Next, we compared the properties of CnL and VhL (Table 1). CnL produces a blue light with a maximum at 465 nm, which was coincident with that of VhL. The K_m value of CnL (30.0 μM) is slightly lower than that of VhL (11.5 μM), whereas their V_{max} values are almost same (2.20×10^5 RLU/s). The half-life of CnL in culture medium at 37°C is 53 h, which is a similar to that of VhL (50 h), indicating that CnL is also a very stable luciferase. These results suggest that CnL is appropriate to use as a reporter enzyme for monitoring of gene expression in living mammalian cells as same as VhL.

Secretion and activity of CnL in cultured mammalian cells

VhL has a secreted property as a convenient reporter of gene expression in living cells, indicating it is distinctly different from non-secreted type reporter enzymes, such as chloramphenicol acetyl transferase, firefly luciferase, and bacterial luciferase.[6] CnL activity in culture medium were much higher than that of VhL in both NIH3T3 (320-fold) and HeLa 3S (410-fold) cells (Fig. 3). This tendency was also confirmed by transient transfection assay using other vectors carrying VhL and CnL, in which these luciferases were expressed under the control of elongation factor-1α or clock gene promoters (unpublished results). Furthermore, we compared the activities of CnL and VhL in culture medium and cell extracts in NIH3T3 and HeLa S3 cells. 94% and 86% of CnL activities were detected in culture medium of NIH3T3 and HeLa cells, respectively (panel A), whereas only 63% and 69% of VhL activities were detected in medium of NIH3T3 and HeLa cells (panel B). These results suggest that CnL is more potent

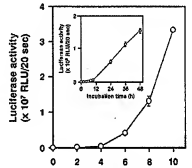


Fig. 4. Continuous Measurement of CnL Activity in the Culture Medium of NIH3T3 Cells until 10 h and until 48 h (inset of figure). The values are plotted as the means±S.D. ($n = 4$).

secreted-reporter enzyme than VhL. Thus, for continuous monitoring of gene expression without destroying cells, the high activity of CnL in the culture medium has advantages that the luciferase activity can be detected rapidly in a small aliquot of culture medium. Indeed, when we continuously measured CnL activity in culture medium of pcDNA–CL-transfected-NIH3T3 cells, the activity was detected within 4 h in the culture medium (3×10^5 RLU/20 s), and the activity increased constantly until 48 h (Fig. 4). Thus, light activity of CnL luciferase can simply trace the promoter activity in the living cell from early time to a long time.

In conclusion, we have cloned a new secreted luciferase CnL from *C. noctiluca* and demonstrated that the activity of CnL in culture medium remarkably higher than that of VhL. This potent of CnL could be benefit to monitoring of gene expression with high sensitive and resolution in living cells.

Acknowledgments

We would like to thank Prof. S. Nagata of Osaka University for providing the pSV2–VL plasmid. We also

thank K. Ogoh of Shizuoka University for samples of *C. noctiluca*. This work was supported in part by the Sumitomo Foundation (to Y. O.), and in part by a Grant-in-Aid for the Support of Young Researchers (no. 13073-2125-14) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to Y. N.).

References

1) Harvey, N., "Bioluminescence", ed. Harvey, N., Academic Press, NY, pp. 297–331 (1952).
2) Johnson, F. H., and Shimomura, O., Introduction to the *Cypridina* system. *Methods Enzymol.*, **57**, 331–364 (1978).
3) Tsuji, F. I., *Cypridina* luciferin and luciferase. *Methods Enzymol.*, 57, 364–372 (1978).
4) Shimomura, O., Johnson, F. H., and Masugi, T., *Cypridina* bioluminescence: light-emitting oxyluciferin–luciferase complex. *Science*, **164**, 1299–1300 (1969).
5) Thompson, E. M., Nagata, S., and Tsuji, F. I., Cloning and expression of cDNA for the luciferase from the marine ostracod *Vargula hilgendorfii*. *Proc. Natl. Acad. Sci. U. S. A.*, 86, 6567–6571 (1989).
6) Thompson, E. M., Nagata, S., and Tsuji, F. I., *Vargula hilgendorfii* luciferase: a secreted reporter enzyme for monitoring gene expression in mammalian cells. *Gene*, 96, 257–262 (1990).
7) Inouye, S., Ohmiya, Y., Toya, Y., and Tsuji, F. I., Imaging of luciferase secretion from transformed Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. U. S. A.*, **89**, 9584–9587 (1992).
8) Tanahashi, Y., Ohmiya, Y., Honma, S., Katsuno, Y., Ohta, H., Nakamura, H., and Honma, K., Continuous measurement of targeted promoter activity by a secreted bioluminescence reporter, *Vargula hilgendorfii* luciferase. *Anal. Biochem.*, **289**, 260–266 (2001).
9) Cohen, A. C., and Morien, J., "Ostracoda and Global Events", eds. Whatley, R., and Maybury, C., Chapman and Hall, Cambridge, pp. 381–400 (1990).
10) Thompson, C. M., Herring, P. J., and Campbell, A. K., Widespread occurrence and tissue distribution of the imidazolopyrazine luciferins. *J. Biolumin. Chemilumin.*, **12**, 87–91 (1997).
11) Haneda, Y., "The Bioluminescence of Biological Systems", ed. Johnson, F. H., The American Association for the Advancement of Science, Washington D.C., pp. 356–358 (1955).
12) Tsuji, F. I., Lynch, R. V., and Stevens, C. L., Some properties of luciferase from the bioluminescent crustacean, *Cypridina hilgendorfii*. *Biochemistry*, 13, 5204–5209 (1974).
13) Varki, A., Biological roles of oligosaccharides. *Glycobiology*, **3**, 97–130 (1993).